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Protection by α-tocopherol of the repair of photosystem II during photoinhibition in *Synechocystis* sp. PCC 6803

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ABSTRACT

α-Tocopherol is a lipophilic antioxidant that is an efficient scavenger of singlet oxygen. We investigated the role of α-tocopherol in the protection of photosystem II (PSII) from photoinhibition using a mutant of the cyanobacterium *Synechocystis* sp. PCC 6803 that is deficient in the biosynthesis of α-tocopherol. The activity of PSII in mutant cells was more sensitive to inactivation by strong light than that in wild-type cells, indicating that lack of α-tocopherol enhances the extent of photoinhibition. However, the rate of photodamage to PSII, as measured in the presence of chloramphenicol, which blocks the repair of PSII, did not differ between the two lines of cells. By contrast, the repair of PSII from photodamage was suppressed in mutant cells. Addition of α-tocopherol to cultures of mutant cells returned the extent of photoinhibition to that in wild-type cells, without any effect on photodamage. The synthesis *de novo* of various proteins, including the D1 protein that plays a central role in the repair of PSII, was suppressed in mutant cells under strong light. These observations suggest that α-tocopherol promotes the repair of photodamaged PSII by protecting the synthesis *de novo* of the proteins that are required for recovery from inhibition by singlet oxygen.

1. Introduction

Exposure of photosynthetic organisms to strong light results in the specific inactivation of photosystem II (PSII) and this phenomenon is referred to as the photoinhibition of PSII [1–4]. In living cells, light-induced damage (photodamage) to PSII and the repair of photodamaged PSII take place simultaneously. Photoinhibition occurs when the rate of photodamage to PSII exceeds the rate of repair of photodamaged PSII. Thus, in order to understand the nature of photoinhibition, it is necessary to monitor the processes of photodamage and repair separately. Methods for the separate monitoring of photodamage and repair have been established in cyanobacteria and plants [5–7] and their application has revealed several new aspects of the mechanisms of photoinhibition [8–14]. When the effects of reactive oxygen species (ROS) on photoinhibition were examined by such methods in cyanobacteria, it became clear that ROS, which include hydrogen peroxide (H_2O_2), the superoxide anion radical ($\bullet O_2^-$), the hydroxyl radical (•OH), and singlet oxygen ($^1O_2$), act primarily by inhibiting the repair of photodamaged PSII rather than by damaging PSII directly [15–17]. This conclusion was supported by studies with *Chlamydomonas* in which the Calvin cycle was impaired [18,19] and, also, by studies with *Arabidopsis* mutants with defects in thermal dissipation [20,21], in photorespiration [22], and in the cyclic transport of electrons via photosystem I [21].

The roles of H_2O_2, $\bullet O_2^-$, and •OH in photoinhibition were demonstrated by monitoring the effects of the addition of H_2O_2 and methyl viologen, a ROS-inducing compound, to suspensions of cyanobacterial cells and, also, the effects of insertional mutagenesis of genes for H_2O_2-scavenging enzymes [15,16]. By contrast, the role of $^1O_2$ in photoinhibition was demonstrated by monitoring the effects of the intracellular production of $^1O_2$ in the presence of exogenous photosensitizers, such as rose bengal and ethyl eosin, as well as by monitoring the effects of semi-anaerobic conditions under which molecular oxygen was largely eliminated from cyanobacterial cells by inclusion of glucose and glucose oxidase in the culture medium [17]. Since these studies fail to exclude the extraneous effects of the added chemicals and the nonspecific effects of the elimination of molecular oxygen, the role in photoinhibition of $^1O_2$ that is intrinsically produced within the cell remains to be fully elucidated.

Abbreviations: Chl, chlorophyll; PSII, photosystem II; ROS, reactive oxygen species

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α-Tocopherol is a particularly efficient scavenger of intracellular $^1O_2$ [23,24]. This antioxidant is localized both in the envelope and in the thylakoid membranes of chloroplasts [25]. Studies with photosynthetic organisms that lack α-tocopherol or those with low levels of α-tocopherol have shown that α-tocopherol protects PSII from photoinhibition [26]. Treatment of *Chlamydomonas* cells with inhibitors of the biosynthesis of α-tocopherol enhanced the photoinhibition of PSII [27–29]. Moreover, *chlP* transgenic lines of tobacco plants with reduced levels of α-tocopherol, carotenoids, and chlorophylls were abnormally sensitive to photoinhibition of PSII [30,31]. The *vte1* mutant of *Arabidopsis*, in which synthesis of α-tocopherol is completely absent, was more sensitive to the photoinhibition of PSII than was the wild-type line [32]. However, none of the cited studies examined whether the protective effects of α-tocopherol occur at the level of photodamage or repair.

In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), α-tocopherol is synthesized via a specific biosynthetic pathway [33–36]. In a mutant of *Synechocystis* that lacks an intact *hpd* gene (*slr0090*), which encodes 4-hydroxyphenylpyruvate dioxygenase, a key enzyme in the biosynthesis of α-tocopherol, no detectable α-tocopherol is produced [33]. Although this enzyme is also involved in the biosynthesis of plastoquinone in plants, neither the level of plastoquinone nor levels of other photosynthetic pigments are affected in the *hpd* mutant [33].

In the present study, we examined the potential role of α-tocopherol in the photoinhibition of PSII using the *hpd* mutant of *Synechocystis*. Mutant cells were more sensitive to photoinhibition than wild-type cells, but the rate of photodamage in the mutant cells was the same as in wild-type cells when the repair of PSII was blocked by chloramphenicol. Our observations suggest that α-tocopherol protects the repair of PSII from inhibition by $^1O_2$.

2. Materials and methods

2.1. Cells and culture conditions

Cells of the glucose-tolerant strain (hereafter referred to as the wild type) and the *hpd* (*slr0090*) mutant [33] of *Synechocystis* sp. PCC 6803 were grown photoautotrophically at 32 °C in liquid BG11 medium under light at 70 μmol photons m^{-2} s^{-1} with aeration by sterile air that contained 1% (v/v) CO_2 [5].

2.2. Assay of the photoinhibition of PSII

Cells in culture at an optical density at 730 nm of 1.0 ± 0.1 were exposed to light at 2000 μmol photons m^{-2} s^{-1} at 25 or 30 °C for designated times to induce the photoinhibition of PSII. For assays of photodamage, chloramphenicol was added to the suspension of cells at a final concentration of 200 μg mL^{-1} just before the onset of illumination. For examination of the effects of exogenous α-tocopherol, a solution of α-tocopherol in ethanol was added to the suspension of cells at a final concentration of 1 mM (0.1% ethanol) with incubation for 5 min prior to illumination. For assays of repair, cells were exposed to 3000 μmol photons m^{-2} s^{-1} at 25 °C without aeration to decrease the activity of PSII to approximately 30% of the initial level. Then cells were transferred to light at 70 or 200 μmol photons m^{-2} s^{-1} and incubated at 25 °C with standard aeration. The activity of PSII was measured at 30 °C in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone and 1 mM $K_3Fe(CN)_6$ with a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, UK) .

2.3. Labeling of proteins in vivo

For pulse labeling of proteins, 25 mL of cell culture was supplemented with 240 kBq mL^{-1} ^{35}S-labeled methionine and cysteine (EasyTag EXPRE^{35}S^{35}S; PerkinElmer, Waltham, MA, USA) and incubated at 25 °C in light at 2000 μmol photons m^{-2} s^{-1}, as described previously [17]. Aliquots of 7 mL each were withdrawn at designated times for analysis of proteins. Labeling was terminated by the addition of nonradioactive methionine and cysteine to a final concentration of 2 mM each with immediate cooling of samples on ice. For the "chase" of pulse-labeled proteins, 25 mL of cell culture was supplemented with nonradioactive methionine and cysteine at a final concentration of 2 mM each after labeling with 80 kBq mL^{-1} ^{35}S-labeled methionine and cysteine for 10 min in light at 2000 μmol photons m^{-2} s^{-1}, and then cultures were incubated at 25 °C for designated times in light at the same intensity, as described previously [17]. Thylakoid membranes were isolated from cells as described previously [17], and membrane proteins were separated by SDS-PAGE on a 15% polyacrylamide gel that contained 6 M urea, as described by Kashino et al. [37]. Labeled proteins on the gel were visualized with an imaging analyzer (FLA-7000; Fujifilm, Tokyo, Japan) and levels of the D1 protein were determined densitometrically. Levels of labeled proteins in thylakoid membranes and in the cytosol were also quantitated by liquid scintillation counting, as described previously [38].

3. Results

3.1. Absence of α-tocopherol enhances the photoinhibition of PSII without affecting the rate of photodamage to PSII

When cells were exposed to light at 2000 μmol photons m^{-2} s^{-1} at 25 °C, the activity of PSII in *hpd* mutant cells disappeared more rapidly than that in wild-type cells (Fig. 1A). After illumination for 120 min, the activity of PSII in mutant cells was approximately half of that in wild-type cells. These observations indicate that the absence of α-tocopherol enhances the extent of photoinhibition of PSII. However, when cells were exposed to light at the same intensity in the presence of chloramphenicol, which blocks the repair of PSII, the activity of PSII in mutant cells declined at the same rate as that in wild-type cells (Fig. 1B). Thus, it appears that the absence of α-tocopherol might not induce photodamage to PSII but might, rather, inhibit the concurrent repair of photodamaged PSII.

When cells were exposed to strong light at a higher temperature, 30 °C, there was slightly less difference in the extent of photoinhibition between wild-type and mutant cells, and the extents of photoinhibition were lower than those at 25 °C (Supplemental Fig. S1). Thus, the combination of strong light and low temperature increased the sensitivity of cells to photoinhibition. In the presence of chloramphenicol, there was, as noted above, no difference in the rate of photodamage at 30 °C between the two strains (Supplemental Fig. S1).

3.2. Addition of α-tocopherol to cultures of the hpd mutant alleviates photoinhibition

To examine whether exogenous α-tocopherol might rescue the phenotype of the mutant cells, we added 1 mM α-tocopherol to cultures of the *hpd* mutant before illumination at 2000 μmol photons m^{-2} s^{-1} at 25 °C. Addition of α-tocopherol resulted in an extent of photoinhibition in mutant cells that was the same as that in wild-type cells (Fig. 2A). However, exogenous α-tocopherol did not affect the rate of photodamage to PSII in the presence of chloramphenicol (Fig. 2B). These observations suggest that exogenous α-tocopherol might enter cells and protect the repair of photodamaged PSII.

3.3. Absence of α-tocopherol inhibits the repair of photodamaged PSII

We examined the effects of the absence of α-tocopherol on the repair of photodamaged PSII. After cells had been exposed to strong light at 3000 μmol photons m^{-2} s^{-1} without aeration, to decrease the activity of PSII to approximately 30% of the initial level, cells were transferred to weak or medium-intensity light and incubated with standard aeration. Under weak light at 70 μmol photons m^{-2} s^{-1}, the

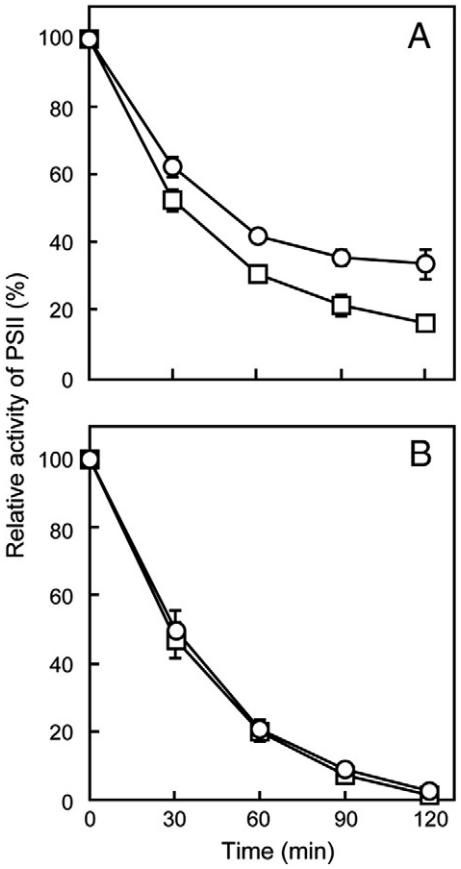

Fig. 1. Effects of the absence of intracellular α-tocopherol on the light-induced inactivation of PSII in *Synechocystis*. Wild-type (○) and *hpd* mutant (□) cells were incubated at 25 °C in light at 2000 μmol photons m^{-2} s^{-1} with standard aeration in the absence of chloramphenicol (A) and in its presence (B). The activity of PSII was monitored in terms of the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. The activities taken as 100% for wild-type and *hpd* mutant cells were 532 ± 132 and 593 ± 191 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Values are means ± SD (bars) of results from three independent experiments.

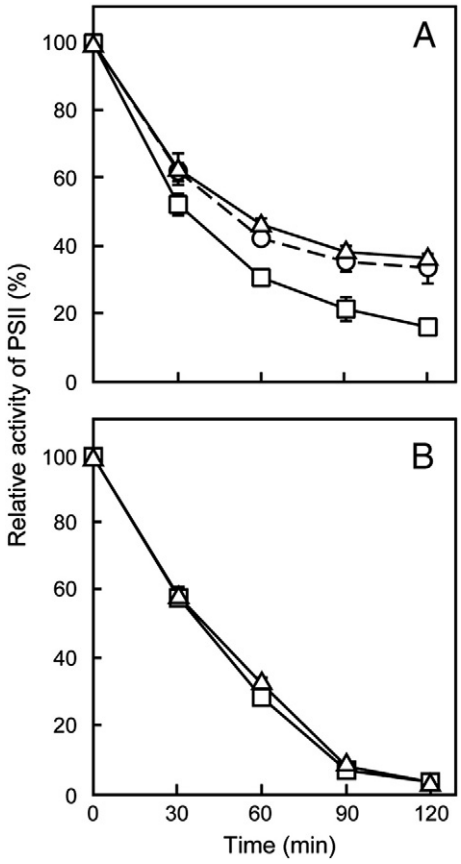

Fig. 2. Effects of addition of α-tocopherol to the culture medium on the light-induced inactivation of PSII in the *hpd* mutant of *Synechocystis*. Cells of the *hpd* mutant were incubated at 25 °C in light at 2000 μmol photons m^{-2} s^{-1}, with standard aeration, in the absence of chloramphenicol (A) and in its presence (B) after the culture medium had been supplemented with 1 mM α-tocopherol. Δ, addition of α-tocopherol; □, no addition of α-tocopherol. The dashed line in (A) shows the results for wild-type cells without the addition of α-tocopherol. The activities taken as 100% for wild-type and *hpd* mutant cells were 468 ± 123 and 485 ± 142 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Values are means ± SD (bars) of results from three independent experiments.

activity of PSII in both wild-type and mutant cells was restored at similar rates (Supplemental Fig. S2). The transfer of cells to weak light might immediately have moderated oxidative conditions within the cells and ROS at levels sufficient to inhibit the repair of PSII might not have been produced in weak light. When cells were transferred to medium-intensity light at 200 μmol photons m^{-2} s^{-1}, the activity of PSII recovered in both types of cell (Fig. 3). However, the rate of recovery in mutant cells was lower than that in wild-type cells, suggesting that absence of α-tocopherol might inhibit the repair of PSII. The smaller difference in repair between the two strains than that in the extent of photoinhibition (Fig. 1) might be due to the production of less amounts of ROS under medium-intensity light.

3.4. Absence of α-tocopherol suppresses the synthesis of the D1 protein de novo

The synthesis of the D1 protein *de novo* plays a central role in the repair of photodamaged PSII [2–4]. To examine the effects of the absence of α-tocopherol on the synthesis *de novo* of the D1 protein, we monitored the incorporation of ^{35}S-labeled methionine and cysteine into proteins during the exposure of cells to strong light at 2000 μmol photons m^{-2} s^{-1}. Fig. 4 shows the patterns of pulse-labeled proteins from thylakoid membranes after SDS-PAGE and time courses of the synthesis of the labeled D1 protein. The rate of synthesis of the D1 protein *de novo* in mutant cells was lower than that in wild-type cells. Thus, it appears that the absence of α-tocopherol suppressed the synthesis of the D1 protein *de novo* during

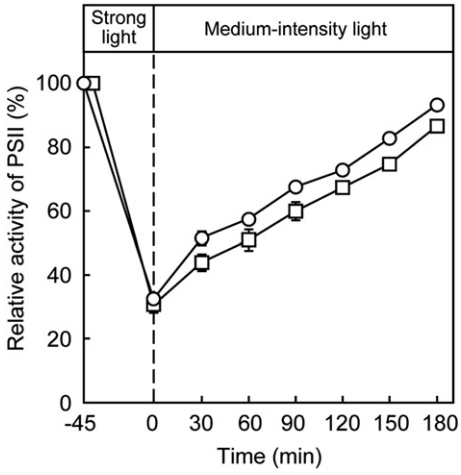

Fig. 3. Effects of the absence of intracellular α-tocopherol on the recovery of PSII from light-induced inactivation in *Synechocystis*. After incubation at 25 °C in light at 3000 μmol photons m^{-2} s^{-1}, without aeration, wild-type (○) and *hpd* mutant (□) cells were transferred to medium-intensity light at 200 μmol photons m^{-2} s^{-1} and incubated at 25 °C with standard aeration. The activities taken as 100% for wild-type and *hpd* mutant cells were 466 ± 93 and 462 ± 125 μmol O_2 mg^{-1} Chl h^{-1}, respectively. Values are means ± SD (bars) of results from three independent experiments.

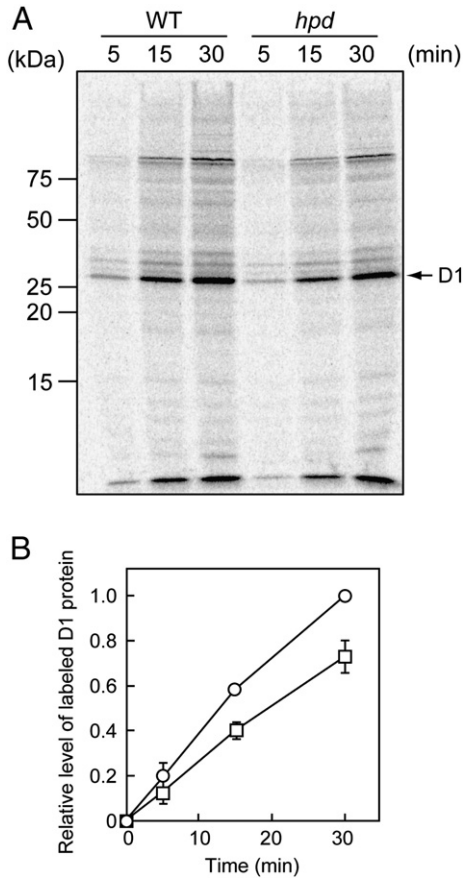

Fig. 4. Effects of the absence of intracellular α-tocopherol on the synthesis of the D1 protein *de novo* in *Synechocystis*. Proteins of wild-type (WT) and *hpd* mutant cells were pulse-labeled by incubation of cells at 25 °C, for the indicated times, in light at 2000 μmol photons m^{-2} s^{-1} in the presence of ^{35}S-labeled methionine and cysteine (see section 2.3 for details). Thylakoid membranes were isolated and proteins were separated electrophoretically. (A) A representative radiogram of pulse-labeled proteins from thylakoid membranes. (B) Quantitation of the levels of the D1 protein in wild-type (○) and *hpd* mutant (□) cells. Values are means ± SD (bars) of results from three independent experiments. The absence of a bar in this and other figures indicates that the bar falls within the symbol.

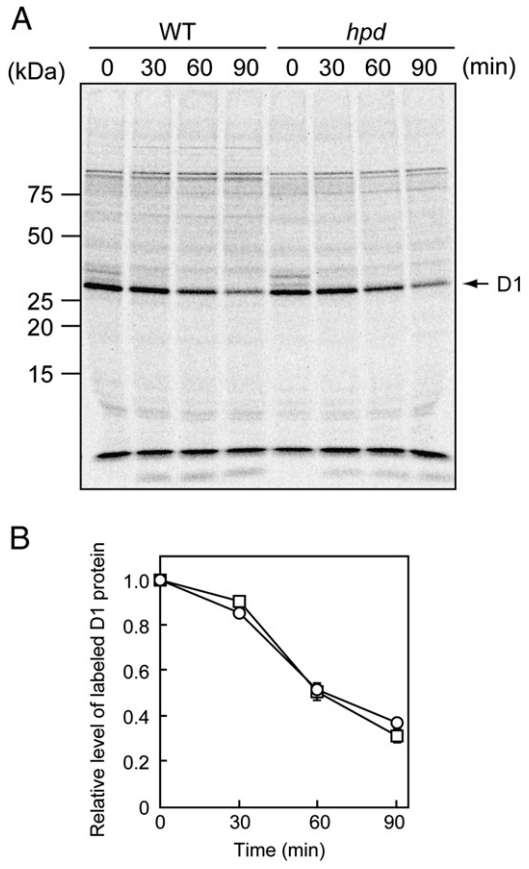

Fig. 5. Effects of the absence of intracellular α-tocopherol on the degradation of the D1 protein in *Synechocystis*. Proteins of wild-type (WT) and *hpd* mutant cells were pulse-labeled by incubation of cells at 25 °C, for 10 min, in light at 2000 μmol photons m^{-2} s^{-1} in the presence of ^{35}S-labeled methionine and cysteine (see section 2.3 for details). Then the suspension of cells was supplemented with nonradioactive methionine and cysteine and incubated for the indicated times under light at the same intensity. Thylakoid membranes were isolated and thylakoid proteins were separated electrophoretically. (A) A representative radiogram showing the results of the "chase" of pulse-labeled proteins from thylakoid membranes. (B) Quantitation of the levels of the D1 protein in wild-type (○) and *hpd* mutant (□) cells. Values are means ± SD (bars) of results from three independent experiments. See legend to Fig. 4 for details.

photoinhibition. By contrast, a "chase" of pulse-labeled proteins showed that there was no difference in terms of the rate of degradation of the D1 protein between mutant and wild-type cells (Fig. 5), suggesting that the absence of α-tocopherol does not accelerate the degradation of the D1 protein during photoinhibition.

3.5. The absence of α-tocopherol suppresses the global synthesis of proteins

Inspection of Fig. 4 revealed that not only the synthesis of the D1 protein but also the synthesis of almost all the proteins of thylakoid membranes was suppressed in the *hpd* mutant cells. We examined the global effects of the absence of α-tocopherol on the synthesis *de novo* of cellular proteins by quantifying pulse-labeled proteins by liquid scintillation counting. Levels of newly synthesized proteins in both the cytosol and thylakoid membranes from mutant cells were lower than those from wild-type cells (Fig. 6). It appears that the absence of α-tocopherol has negative effects on the global synthesis of proteins during strong illumination.

4. Discussion

4.1. α-Tocopherol protects the repair of PSII during photoinhibition

The present study demonstrated that, in *Synechocystis*, the absence of α-tocopherol enhances the extent of photoinhibition of PSII not by inducing photodamage to PSII but, rather, by inhibiting the repair of photodamaged PSII. Strong sensitivity to photoinhibition due to the complete lack or low levels of α-tocopherol has been observed in several studies [26–32], but none of the cited studies examined the effects of α-tocopherol with the separation of photodamage from repair. The present study revealed that it is the process of repair that is protected by α-tocopherol during the photoinhibition of PSII.

The general effects of the absence of α-tocopherol appear, however, to be relatively modest in *Synechocystis*. The limited extent of its effects might be due to induction of mechanisms that compensate for the lack of α-tocopherol. In the *vte1* mutant of *Arabidopsis*, which lacks α-tocopherol, increased levels of carotenoids and the consequent induction of thermal dissipation compensate for the absence of α-tocopherol and alleviate the enhanced sensitivity to photoinhibition [32]. By contrast, the marked sensitivity to photoinhibition of *chlP* transgenic lines of tobacco plants might be due to the synergistic effects of decreased levels of both α-tocopherol and carotenoids [30,31]. In addition, molecular chaperons might also compensate for the lack of α-tocopherol, since HtpG, a homolog of Hsp90, participates in the protection of cells from oxidative stress and is inducible under oxidative conditions in cyanobacteria [39,40].

Addition of α-tocopherol to cultures of *hpd* mutant cells restored photoinhibition to the level in wild-type cells without affecting the photodamage to PSII (Fig. 2). It seems likely that exogenous α-

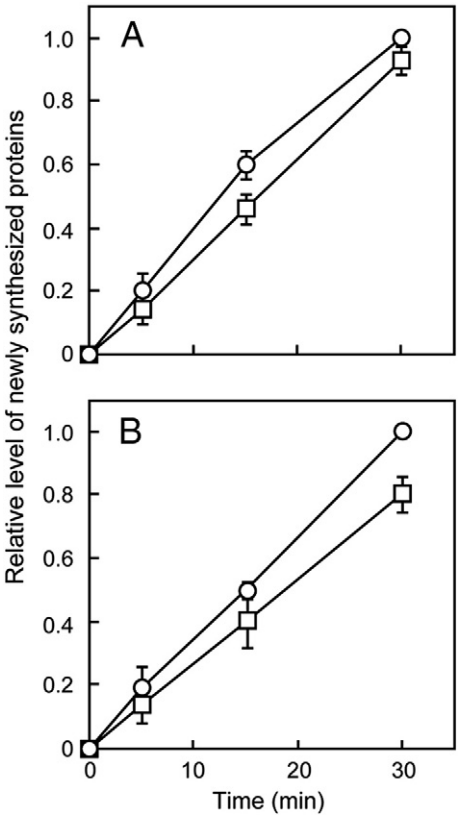

Fig. 6. Effects of the absence of intracellular α-tocopherol on the global synthesis of proteins *de novo* in *Synechocystis*. Proteins in wild-type (○) and *hpd* mutant (□) cells were pulse-labeled by incubation of cells at 25 °C, for the indicated times, in light at 2000 μmol photons m^{-2} s^{-1} in the presence of ^{35}S-labeled methionine and cysteine (see section 2.3 for details). Proteins from thylakoid membranes (A) and the cytosol (B) were fractionated and the levels of labeled proteins in both fractions were determined by liquid scintillation counting. Values are means ± SD (bars) of results from three independent experiments.

tocopherol might enter cells and accumulate in the thylakoid membranes of *Synechocystis*. By contrast, only tocopherol derivatives with short chains can penetrate *Chlamydomonas* cells [29]. The accumulation of exogenously added α-tocopherol in the thylakoid membranes of *Synechocystis* might compensate for the absence of α-tocopherol in the mutant cells and protect the repair of PSII in these cells.

4.2. α-Tocopherol protects the synthesis of the D1 protein de novo

Pulse labeling of proteins *in vivo* demonstrated that the absence of α-tocopherol suppresses the synthesis of the D1 protein *de novo* during photoinhibition (Fig. 4). Rapid decreases in levels of the D1 protein were observed during photoinhibition in *Chlamydomonas* cells that had been treated with inhibitors of the biosynthesis of α-tocopherol [27–29] and, also, in the *vte1* mutant of *Arabidopsis* [32]. The authors of the studies with *Chlamydomonas* attributed the decreased level of the D1 protein to accelerated degradation of the D1 protein that was a consequence of reduced levels of α-tocopherol. However, the "chase" of labeled proteins, in the present study, revealed that the absence of α-tocopherol does not accelerate the degradation of the D1 protein during photoinhibition in *Synechocystis* (Fig. 5). Thus, the rapid decrease in the level of the D1 protein was due to low rate of synthesis of the D1 protein *de novo* in the absence of α-tocopherol. Since the D1 protein plays a central role in the repair of PSII, it appears that α-tocopherol protects the repair of PSII by enhancing the synthesis of the D1 protein *de novo* during exposure of *Synechocystis* to strong light. Moreover, the global suppression of protein synthesis in the absence of α-tocopherol might further inhibit the repair of PSII via adverse effects on the synthesis of a variety of proteins that are involved in the repair system, in addition to the D1 protein.

4.3. Action of $^1O_2$ in the photoinhibition of PSII

Since α-tocopherol acts as an efficient scavenger of $^1O_2$ [23,24], it is likely that the absence of α-tocopherol results in the accumulation of excessive amounts of $^1O_2$ within cells under strong light. Inhibition of the repair of PSII in the absence of α-tocopherol suggests that $^1O_2$ might act by inhibiting the repair of PSII during photoinhibition. Furthermore, the finding that the absence of α-tocopherol does not affect the rate of photodamage suggests that $^1O_2$ might not act by damaging PSII directly during photoinhibition. These observations are consistent with observations of the action of $^1O_2$ that is produced by photosensitizers: production of excess $^1O_2$ within cells in response to photosensitizers, such as rose bengal and ethyl eosin, does not accelerate photodamage to PSII but, rather, inhibits the repair of photodamaged PSII [17]. The present study, exploiting the natural accumulation of $^1O_2$ within mutant cells, strongly supports the hypothesis that $^1O_2$ acts exclusively by inhibiting the repair of PSII during photoinhibition. Studies of the intracellular production of other ROS, such as H_2O_2 and $\bullet O_2^-$, have also demonstrated that these ROS act by inhibiting the repair of PSII and not by damaging PSII [15,16].

4.4. Action of $^1O_2$ in the inhibition of protein synthesis

Suppression of synthesis of the D1 protein in the absence of α-tocopherol suggests that $^1O_2$ might act by inhibiting the synthesis of the D1 protein *de novo* during photoinhibition. The suppression of the synthesis the D1 protein *de novo* might, in turn, result in inhibition of the repair of PSII. This hypothesis is consistent with the previous finding that intracellular production of $^1O_2$ in response to photosensitizers inhibits the synthesis of the D1 protein at the elongation step of translation under strong light [17]. The global suppression of protein synthesis in the absence of α-tocopherol suggests that $^1O_2$ might inactivate the protein-synthetic system either directly or indirectly. A recent study, using a translation system *in vitro* derived from *Synechocystis*, has demonstrated that H_2O_2 inactivates the translational machinery via oxidation of elongation factor G, a key protein in translational elongation, thereby, inhibiting the synthesis of the D1 protein [38]. The oxidation of elongation factor G by H_2O_2 is due to formation of an intramolecular disulfide bond between specific cysteine residues [41]. It remains to be determined whether $^1O_2$ also inactivates a specific component of the translational machinery.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2010.11.003.

Acknowledgments

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